

Assessment of Within-group Variation in CYP1A mRNA Inducibility in Environmentally Exposed and Chemically Treated Atlantic Tomcod

Simon Courtenay,¹ P. James Williams,¹ Cheryl Grunwald,² Blake Konkle,² Tun-Liang Ong,² Isaac I. Wirgin²

¹Canadian Department of Fisheries and Oceans, Moncton, New Brunswick, Canada; ²Nelson Institute of Environmental Medicine, New York University Medical Center, Tuxedo, New York

CYP1A gene expression has been implicated in the processing of environmental procarcinogens and levels of variation in *CYP1A* mRNA expression are high in both environmentally exposed and chemically treated Atlantic tomcod. The objective of this study was to evaluate the effects of physical and biological parameters such as temperature, sex, and reproductive state on within-group variation in *CYP1A* mRNA induction. Levels of variation in *CYP1A* mRNA expression were directly correlated with mean levels of gene induction. Our results indicate that sex and reproductive state, but not temperature, had significant effects on *CYP1A* mRNA inducibility in tomcod; however, these parameters did not account for all interindividual variation in *CYP1A* inducibility. Other intrinsic biological factors, such as genetic polymorphisms in molecular pathways leading to *CYP1A* induction, may contribute to the high levels of interindividual variation in *CYP1A* inducibility in Atlantic tomcod. — Environ Health Perspect 102(Suppl 12):85–90 (1994)

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Hepatic Neoplasia in Hudson River Tomcod

Individuals within natural populations exhibit differing susceptibilities to diseases such as cancer. For example, a high percentage (50–90%), although not all, of Atlantic tomcod (*Microgadus tomcod*) from the chemically impacted Hudson River exhibit liver tumors, whereas the prevalence of neoplasia is much lower (<5%) in tomcod from other rivers along the North American Atlantic coast (1,2). While this difference in the prevalence of neoplasia among populations almost certainly has an environmental component, evidence suggests that genetic variation or an interaction of heritable variation in susceptibility

and exposure may also be responsible. Genetic differences in disease susceptibility may be reflected at the species, population, or individual level. For example, within the Hudson River, at the species level, only tomcod exhibit a high prevalence of hepatic neoplasms whereas other bottom-dwelling finfish species which share a common ecological niche show no evidence of cancer.

Variation in the prevalence of neoplasia at these levels of biological organization may result from significant differences in exposure histories to contaminants, physical factors, differences in biological parameters such as sex, growth rate, or variation at carcinogenically relevant genetic loci. Genetic variation may result directly from the genotoxic actions which are a consequence of exposure to xenobiotic agents or from heritable genetic polymorphisms whose frequencies differ among species, populations, or individuals. Genetic variation at these loci may involve mutations in coding regions of genes, which determine the structural properties of protein products or from polymorphisms in their regulatory regions, which in turn may impact on levels of gene expression. For example, many studies have shown that somatic mutations in the *ras* oncogenes accompany the initiation of

neoplasia in humans, carcinogen-exposed animal models, and chemically (3) and environmentally exposed (4,5) fish.

CYP1A Inducibility

In this regard, recent studies in humans have investigated the impact of heritable polymorphisms in cytochrome P450 genes on susceptibility to various cancers. Initial evidence suggests that mutations in the coding regions (6) and introns (7) of cytochrome P450's influence susceptibility to some cancers. Studies have also demonstrated significant variation in *CYP1A* inducibility among individuals, perhaps resulting from polymorphisms in *CYP1A* regulatory regions or other genes implicated in pathways leading to *CYP1A* transcription or translation (8). Observations have led to the suggestion that differences in gene inducibility may also play a role in disease susceptibility. However, in human studies, because of the difficulty in controlling for factors such as exposure history, age, and sex, it is also difficult to determine the effects of among individual genetic variation on the inducibility of the *CYP1A* gene.

CYP1A Induction in Atlantic Tomcod

CYP1A monooxygenase enzymes convert exogenous environmental procarcinogens

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Address correspondence to Dr. Isaac I. Wirgin, Nelson Institute of Environmental Medicine, New York University Medical Center, Long Meadow Road, Tuxedo, NY 10987. Telephone (914) 351-2415. Fax (914) 351-5472.

to diol epoxides, a form in which they can adduct to DNA at carcinogenically significant genetic loci. Levels of CYP1A mRNA in exposed and unexposed Atlantic tomcod may provide a unique opportunity to quantify and assess the impact of genetic variation on interindividual differences in inducibility at this carcinogenically relevant gene locus. In controlled laboratory experiments, we have demonstrated that CYP1A mRNA is inducible in Atlantic tomcod by exposure to low concentrations of aromatic hydrocarbons such as PAHs, a coplanar PCB congener, and TCDD and that levels of gene expression are dose-responsive (Grunwald et al., unpublished data). Additionally, CYP1A mRNA expression in environmentally exposed tomcod from the Hudson River was significantly higher than that seen in tomcod from four other North Atlantic rivers (9), although prior exposure history to chlorinated aromatic hydrocarbons may significantly affect CYP1A mRNA inducibility (10). Bleached kraft mill effluent also significantly induced CYP1A gene expression in tomcod caged in the Miramichi River, New Brunswick (11). Clearly, CYP1A mRNA induction in Atlantic tomcod is highly sensitive to environmentally relevant concentrations of several xenobiotics. Furthermore, tomcod from the Hudson River exhibited a transcribed genetic polymorphism in CYP1A which is absent in tomcod from other more northern river systems [(12) Wirgin et al., unpublished data].

The feature common to all these studies on Atlantic tomcod is the high level of interindividual variability in CYP1A mRNA expression. Environmentally exposed tomcod collected from the same site on the same day, or fish caged at a single site, exhibited considerable differences in levels of CYP1A mRNA. Even tomcod exposed to model chemicals under controlled laboratory conditions demonstrated high levels of interindividual variability in CYP1A mRNA induction. We hypothesize that the high level of interindividual variability in levels of CYP1A mRNA among chemically and environmentally exposed fish reflects underlying genetic variation in CYP1A regulation or differences in other genetic factors in the pathway culminating in CYP1A induction. In this study our objectives were 3-fold: to compare the magnitude of variation in basal and induced levels of CYP1A mRNA expression in chemically and xenobiotically exposed Atlantic tomcod; to evaluate the contribution of certain environmental and biological parameters on variation in

CYP1A mRNA inducibility in tomcod; and to assess the impact of genetic factors on variation of CYP1A inducibility in tomcod. Our strategy in this study was to initially quantify overall levels of variation in CYP1A mRNA levels within groups of environmentally exposed, unexposed, or chemically treated tomcod. We then evaluated the impact of those parameters which have been previously shown to influence basal or induced levels of CYP1A gene expression including temperature, sex, reproductive status, and level of exposure. By determining the impact of these parameters on levels of variation in CYP1A gene expression, we obtained an initial evaluation of the impact of intrinsic biological and genetic factors on CYP1A gene inducibility.

Methods

Environmentally exposed tomcod were collected from the Hudson, Margaree, Miramichi, Saco, and St. Lawrence rivers or caged in the Miramichi River and immediately sacrificed. These rivers range from highly contaminated (Hudson River) with organic pollutants (PAHs, PCBs, dioxins) to relatively pristine (Margaree River). Chemically treated tomcod were collected from the Hudson or Miramichi rivers, depurated in clean laboratory water for at least 20 days, and injected ip with single doses of the following chemicals (listed in Table 1): beta-naphthoflavone (β -

NF), benzo[a]pyrene (B[a]P), 3,3',4,4'-tetrachlorobiphenyl (TCB), or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Tomcod were injected ip with single doses of these chemicals and they were sacrificed at those times listed in Table 1. Sex, total length, reproductive status, and time of collection and treatment were recorded for each fish when possible. Following sacrifice, livers were removed from all samples and immediately frozen at -80°C .

Total RNA was isolated by the RNazol method (13) and purity and concentration were determined by UV spectrophotometric analysis. Relative CYP1A mRNA concentrations for each sample were determined by northern blot analysis. Total RNAs were separated electrophoretically in denaturing 1.0% formaldehyde agarose gels (14) and RNA integrity was evaluated by ethidium bromide staining. Degraded RNAs were omitted from statistical analysis. RNA was transferred to nylon membranes (15), filters were vacuum baked, prehybridized and hybridized at 65°C (16) to a ^{32}P radiolabeled (17) Atlantic tomcod, β -NF induced, full length (2.6 kb) CYP1A cDNA probe. Final wash conditions were $1.0 \times \text{SSPE}/0.1\%$ SDS at 65°C for 30 min. Membranes were exposed to X-ray film and CYP1A mRNA concentrations were determined using the Whole Band Analysis package of the Millipore Bio Image computer analysis system. To ensure standardized loading of

Table 1. Characteristics of chemical treatments in laboratory and field exposures of Atlantic tomcod.

Experiment	Injection	Exposure period	Groups	N/Group
Chemical treatments				
2,3,7,8-TCDD	500 ppb	9–168 hr	9	5–8
2,3,7,8-TCDD	0 & 500 ppb	2–25 days	9	6–15
2,3,7,8-TCDD	0–5000 ppb	10 days	7	7–24
3,3',4,4'-TCB	0 or 1 ppm	1–10 days	7	7–16
3,3',4,4'-TCB	0–10 ppm	7 days	6	5–8
BNF	100 ppm	4–168 hr	6	4–14
BNF	0–500 ppm	3 days	7	3–6
BNF	0 & 100 ppm	3 days	8	5–10
B[a]p	0 & 10 ppm	8–168 hr	8	4
B[a]p	0–50 ppm	3 days	10	3–6
B[a]p	10 ppm	2 days	6	8–33
Total:11			83	598
Field exposures				
St. Lawrence River	—	—	1	48
Margaree River	—	—	1	45
Hudson River	—	—	4	11–51
Miramachi River	—	—	5	10–45
Saco River	—	—	1	16
Miramachi River (Gender/season)	—	—	5	9–31
Total:6			17	397

Abbreviations: 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCB, 3,3',4,4'-tetrachlorobiphenyl; BNF, β -naphthoflavone; B[a]P, benzo[a]pyrene.

mRNA in each lane, membranes were rehybridized to a housekeeping gene, rRNA. The *CYP1A* probe was removed by incubating membranes in boiling water and then membranes were hybridized to a rat 18S rRNA probe (18) exactly as described above.

Results

High Levels of Variability in *CYP1A* Expression

High levels of variability in *CYP1A* mRNA were observed within environmentally exposed and treated groups (Figure 1). As a result, we sought to determine if this variation reflected intrinsic differences among fish in *CYP1A* mRNA inducibility. Initially we plotted the variance versus the means of *CYP1A* mRNA levels within groups of tomcod exposed via the two different routes; field collected (Figure 1A) and those treated under controlled laboratory conditions (Figure 1B). The relation-

ship between mean and variance was examined within 17 groups of field-exposed fish and 83 groups of fish treated in the laboratory with the four model chemicals or vehicle-injected controls. For the two exposure routes, the magnitude of variation was significantly positively correlated with the mean. For example, tomcod from the more polluted Hudson and Miramichi rivers showed greater variation in gene expression than fish from less polluted rivers (the Margaree and Saco). Under controlled laboratory conditions, variance also showed a significant positive relationship with mean response and thus treatment dose or time of maximum induction.

To determine if the extent of within group variation in *CYP1A* gene expression differed significantly between the routes of exposure, the slopes of the two regression lines were compared by analysis of covariance. Similar slopes of the two lines (1.57 and 1.51) for the field and chemically exposed fish indicate a similar relationship

between variance and means of the field and laboratory samples ($F[1,96]=0.05$, $p=0.823$). But the elevation (i.e., Y -intercept) of the regression line for the field-exposed fish was significantly higher than that for the chemically exposed tomcod ($F[1,97]=11.468$, $p=0.001$), indicating that at any given level of induction, field samples showed greater variance than groups of fish exposed under controlled laboratory conditions. However, these results indicate that variation in *CYP1A* mRNA gene expression was high in tomcod even when treated under controlled laboratory conditions, suggesting that a major component of variation in gene expression in environmentally exposed fish is due to intrinsic biological factors which impact on gene inducibility. These might include biological factors such as sex, reproductive status, or inherent genetic differences among individuals.

Effects of Temperature

The effect of temperature on *CYP1A* mRNA induction was investigated by injecting (ip) Hudson River tomcod with a single treatment of β -NF (100 ppm) and maintaining groups of five to ten fish at 0°, 5°, 7.5°, or 10°C, for 3 days before sacrificing. Controls were injected with corn oil vehicle and similarly held. Two-way analysis of variance ($\ln [X+1]$ transformed data) revealed significant *CYP1A* mRNA induction by β -NF ($F[1, 41] = 70.04$, $p<0.001$) but no significant effect of temperature on the magnitude of induction ($F[3,41] = 2.09$, $p=0.115$). This result is similar to that observed in killifish (*Fundulus heteroclitus*) in which *CYP1A* mRNA expression was not influenced by temperature, although expression of the protein was strongly impacted (19).

Effects of Sex and Reproductive Status

Our data allowed us to systematically evaluate the effects of two biological factors, sex and reproductive status, which have previously been implicated as impacting on *CYP1A* protein expression in fishes and other organisms (20). If significant variation in *CYP1A* gene inducibility is still manifest after these factors have been considered, that would suggest that other factors such as genetic variation may influence gene inducibility.

Figure 2 shows the effects of reproductive status on mean levels and variation of *CYP1A* mRNA expression in environmentally exposed female and male tomcod, respectively, collected from a single site during different seasons, from the industri-

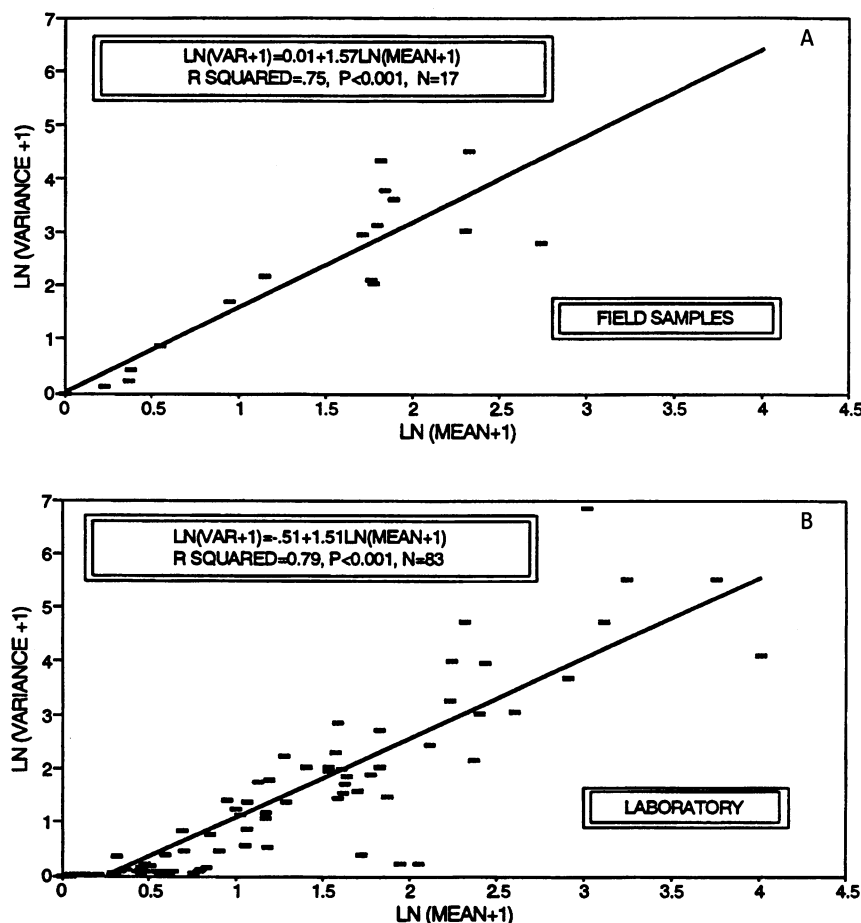


Figure 1. Relationship between variance and mean *CYP1A* responses in groups of Atlantic tomcod sampled from the field (A) and exposed to chemicals in the laboratory (B). Details of treatments are given in Table 1. Lines represent simple linear regressions.

alized Miramichi River. For both sexes, reproductive status significantly affected levels of *CYP1A* gene expression; however, these effects differed between sexes. Pre-spawning females collected in late November showed little evidence of *CYP1A* mRNA expression, whereas levels were high in pre-spawning males. Spent females collected in late January showed high levels of gene expression, while spent males exhibited much lower levels of *CYP1A* mRNA. At spawning time, levels of intact *CYP1A* mRNA were low in both sexes. Initial evidence suggests that post-transcriptional processing of *CYP1A* mRNA may have accounted for this decrease. For both sexes, the level of variation in *CYP1A* mRNA was once again highly correlated with mean levels of gene expression ($R^2 = 0.98-0.99$). From this analysis we conclude that both gender and reproductive status affect levels of *CYP1A* mRNA inducibility in exposed Atlantic tomcod.

However, even when the effects of biological and physical parameters such as sex, season, and reproductive status are accounted for, there is still significant variability in *CYP1A* mRNA inducibility among tomcod. This is apparent by examining the individual data points in Figure 2 A and B. With the exception of pre-spawning females and spawning males, tomcod show high levels of variation in *CYP1A* mRNA expression. From this analysis, we conclude that genetic variation or other intrinsic biological parameters that were not measured in these studies must contribute to high levels of variation in *CYP1A* mRNA inducibility among tomcod.

To gain initial insights into genetic mechanisms perhaps contributing to this variability in *CYP1A* mRNA, a frequency distribution of inducibility was examined in chemically treated tomcod (Figure 3). In these experiments, Hudson River tomcod matched for sex, reproductive state, and season were injected ip with B[a]P.

Although variability in gene induction was high among these fish, responses of each sex did not fall into clearly differentiated modality groupings.

Our results indicate that levels of variation in *CYP1A* mRNA expression are high in both environmentally exposed and chemically treated Atlantic tomcod, although little variability was seen in basal levels of gene expression. Our analyses suggest that variation in levels of induced *CYP1A* mRNA are almost as high in tomcod treated with model chemicals under controlled laboratory conditions as seen in environmentally exposed fish. As a result, we conclude that a major component of variation in *CYP1A* mRNA levels in environmentally exposed tomcod is due to inherent differences in *CYP1A* mRNA inducibility among individuals or groups of fish.

Genetic Variability

We have also demonstrated that sex and reproductive status have a significant impact on inducibility of *CYP1A* mRNA in Atlantic tomcod. For example, pre-spawning exposed females exhibited no induction, whereas pre-spawning exposed males were highly induced. Similar results have been reported in environmentally exposed winter flounder (*Pseudopleuronectes americanus*), where hepatic levels of cytochrome P450 protein were approximately 8-fold lower in gonadally mature females than in gonadally mature males (21) while in a second study at a different exposure site, EROD (ethoxyresorufin-*O*-deethylase) was only two fold higher in sexually mature males than females (22). Even within spawning groups of a single sex, we observed significant differences between ripe and spent fish. However, if the effects of these biological parameters are discounted, high levels of interindividual variation in *CYP1A* mRNA inducibility are still evident. As a result, we conclude this variation is likely due to genetic differences in *CYP1A* mRNA inducibility and other possible biological factors.

Clark and co-workers (8) have reported that EROD activity in TCDD-treated cultured human lymphocytes also exhibits considerable variability in levels of gene expression and responses fall into a bimodal or trimodal distribution, with 70% of individuals categorized as low responders and 30% as high responders perhaps due to genetic differences in *CYP1A* inducibility. While tomcod exhibited high levels of variability in *CYP1A* gene expression we did not detect any evidence of a modal distribution of responses (Figure 3). Furthermore,

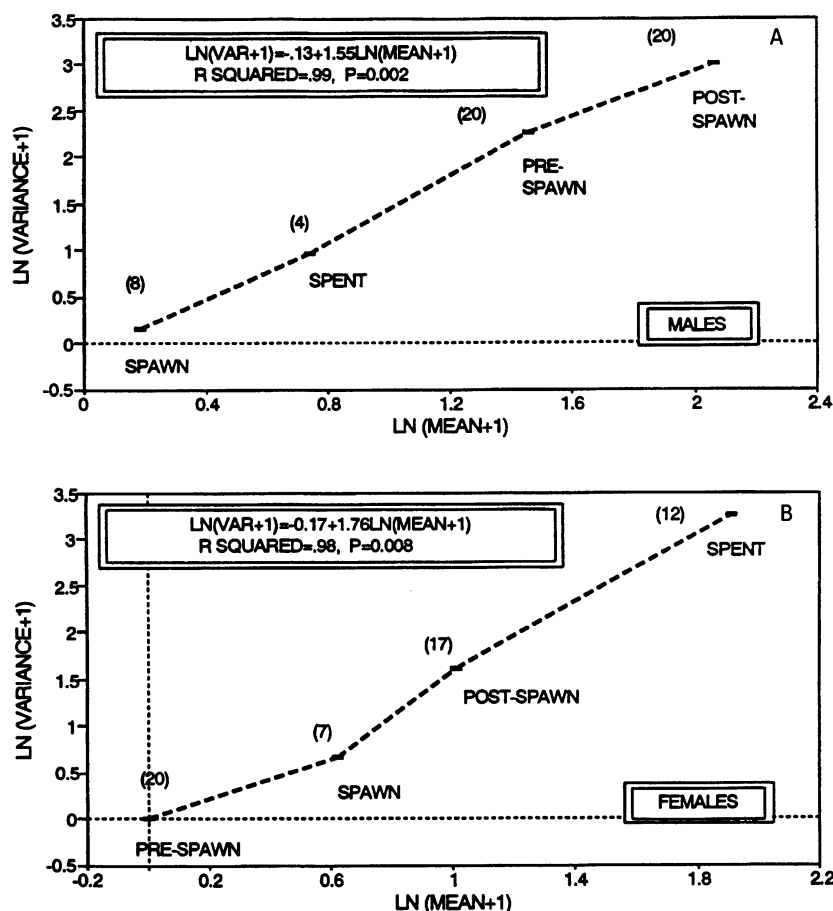


Figure 2. Effect of gender and reproductive status on *CYP1A* mRNA mean level and variance in Atlantic tomcod collected from the Miramichi River. Number above points represent the number of fish sampled.

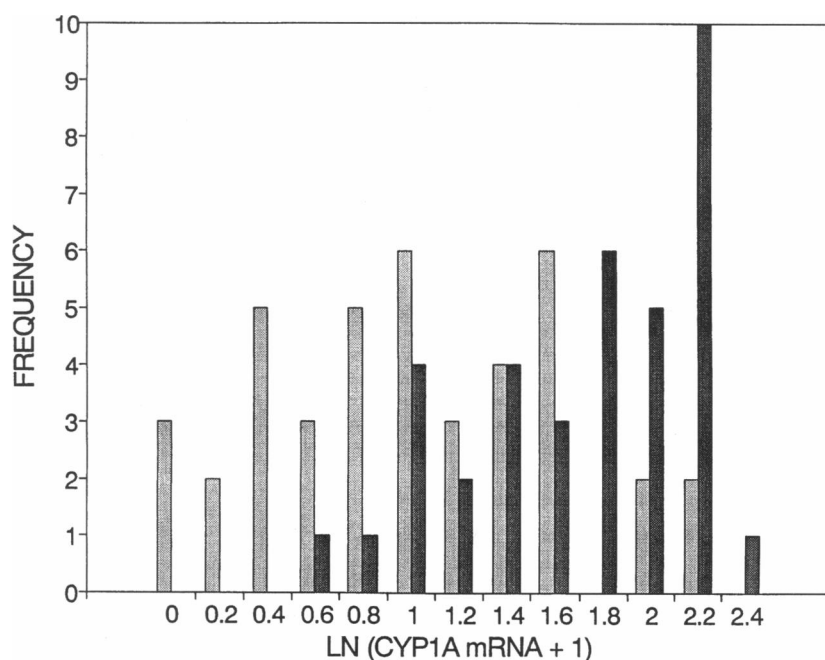


Figure 3. Frequency distribution of CYP1A mRNA levels in Hudson River Atlantic tomcod exposed for 2 days to 10 ppm benzo[a]pyrene. Lightly shaded bars are males collected at Garrison ($n = 41$). Dark bars are females collected at Manhattan ($n = 37$).

we have demonstrated that variation in *CYP1A* inducibility in tomcod results from *in vivo* exposure at environmentally relevant concentrations of inducers and is at the

transcriptional level. However, we have yet to demonstrate concordant variation in *CYP1A* protein levels and enzyme activity among individual fish.

It has been hypothesized that variation in *CYP1A* inducibility in humans may be associated with unspecified polymorphisms at the aromatic hydrocarbon locus (Ah receptor) (23) or a restriction fragment length polymorphism (RFLP) in 3' noncoding sequences of the *CYP1A* gene (7). Additionally, there is evidence that this *CYP1A* RFLP may be associated with increased susceptibility to lung cancer in some racial groups, although not others (24). Although the significance of interindividual variation in *CYP1A* mRNA inducibility in Atlantic tomcod has yet to be demonstrated, our results indicate that the magnitude of this variation is large. Both a genetic component and other biological factors impact on this variation in gene inducibility. We suggest that the magnitude of variation seen in *CYP1A* gene inducibility may be representative of that exhibited by other environmentally responsive genes and that this variation in expression may play a significant role in genotoxic, teratogenic, or acute responses of organisms to environmental insult. Genetic variation in regulation of xenobiotically responsive genes should be considered when evaluating the susceptibility of individuals or populations to contaminant exposure.

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